Primary Characterization of a Monoclonal Antibody Using Agilent HPLC-Chip Accurate-Mass LC/MS Technology

The characterization of a monoclonal antibody at low nanogram levels using an Agilent HPLC-Chip system coupled to an Agilent Accurate-Mass Q-TOF MS instrument is described. The superior sensitivity and mass accuracy of the HPLC-Chip LC/MS platform, combined with the powerful data processing capabilities of Agilent MassHunter and BioConfirm software, enabled easy and rapid identification of antibody heterogeneity and cleaved fragments. Robust, reliable, and easy to use, Agilent nanoflow LC/MS technology is ideally suited for the routine analysis of biopharmaceuticals.

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Introduction

Recombinant monoclonal antibodies (mAbs) represent an important class of biopharmaceutical products with a wide range of diagnostic and clinical applications. The worldwide market for monoclonal antibodies is projected to reach $26 billion by 2010–2011. These therapeutic glycoproteins are usually produced from mammalian cell lines (hybridomas), then purified, concentrated, and exchanged into an appropriate formulation buffer prior to being sold. Although mAbs are relatively stable biomolecules, a number of chemical modifications and degradation reactions can occur during manufacturing, formulation, and storage, thereby necessitating reliable and sensitive methods for the characterization of protein purity and structural integrity. Accurate mass measurements of intact proteins, whole subunits, or domains are useful for the rapid verification of sequence composition and identification of post-translational modifications and sample handling artifacts.

LC/MS technology is a powerful and sensitive technique for the characterization and identification of proteins. Nanoflow LC/MS enables faster and more sensitive protein identification while minimizing sample and solvent consumption. Agilent's microfluidic-based HPLC-Chip integrates sample preparation, chromatographic separation, and nanoelectrospray formation for efficient, high-sensitivity nanospray LC/MS. Agilent Accurate-Mass LC/MS TOF and Q-TOF systems deliver exceptional mass resolution,

mass accuracy, sensitivity, and data processing capabilities for optimal MS characterization of proteins. In this note, we report the rapid LC/MS characterization of low nanogram levels of an intact mAb and its fragments using the Agilent HPLC-Chip system coupled to an Accurate-Mass Q-TOF.

Experimental

Materials

L-cysteine, EDTA disodium, sodium phosphate dibasic anhydrous, sodium phosphate monobasic, sodium bicarbonate, papain suspension (in 0.05 M sodium acetate), and DL-dithiothreitol (DTT) were purchased from Sigma. PNGase and the Tris reaction buffer pH 8.2 were obtained from Prozyme.

Deglycosylation reaction

Deglycosylation of the mAb was performed using PNGase in 20 mM Tris-HCl buffer pH 8.2 as described by the manufacturer. Briefly, 0.3 µL of 100 U/mL solution of PNGase was added to 3 µg of antibody in 20 mM Tris-HCl buffer pH 8.2. The mixture was incubated at 37°C for 24 hr. Samples were appropriately diluted in 3% acetonitrile/water with 0.1% formic acid solution prior to LC/MS analysis.

Papain digestion

The papain was activated using freshly prepared activation buffer (papain activation/digestion buffer: 1 mM EDTA, 50 mM sodium phosphate buffer and 10 mM cysteine, pH 7.0) for 15 min at 37°C. Activated papain solution was then added to the mAb in papain digestion buffer. The mAb-to-protease ratio was 60:1(w/w). The mixture was incubated at 37°C for 2 hr. Samples were appropriately diluted in 3% acetonitrile/water with 0.1% formic acid solution prior to LC/MS analysis.

Reduction reaction

The mAb (1.5 µg) was reduced with 25 mM DTT at pH 8.6 for 1 hr at 80°C. Samples were appropriately diluted in 3% acetonitrile/water with 0.1% formic acid solution prior to LC/MS analysis.

LC/MS Analysis

Instrumentation

The Agilent 1200 HPLC-Chip system was coupled with the Agilent 6520 Accurate-Mass Q-TOF LC/MS platform for LC/MS analyses **(Figure 1)**. The HPLC-Chip is a polymer microfluidic-based device that integrates sample preparation (enrichment column), analytical separation (analytical column), and nano-electrospray formation (emitter tip). The enrichment column helps in efficient desalting and concentration of the sample, reducing sample consumption. The HPLC-Chip Cube interface enables automatic chip loading, sample and solvent delivery to the chip, high pressure switching of flows, and automated chip positioning to the MS source. The Agilent 6520 Q-TOF LC/MS system incorporates True High-Definition TOF technology to provide optimal sensitivity, mass accuracy, mass resolution, and wide dynamic range.

LC parameters

HPLC-Chip: 5 µm, ZORBAX 300SB-C8 (300Å), 40 nL enrichment column, and a 75 µm x 43 mm analytical column.

Flow rate: 3 µL/min from capillary pump to the enrichment column and 300 nL/min from nano LC pump to the analytical column.

Solvents: 0.1% formic acid in water (A); 90% acetonitrile in water with 0.1% formic acid (B). Flush volume was set at 7 µL for effective desalting of the mAb sample.

Sample load: 7.5-10 ng on-column for intact and deglycosylated mAb while 5 ng on-column was used for reduced and papain-generated fragments of the mAb.

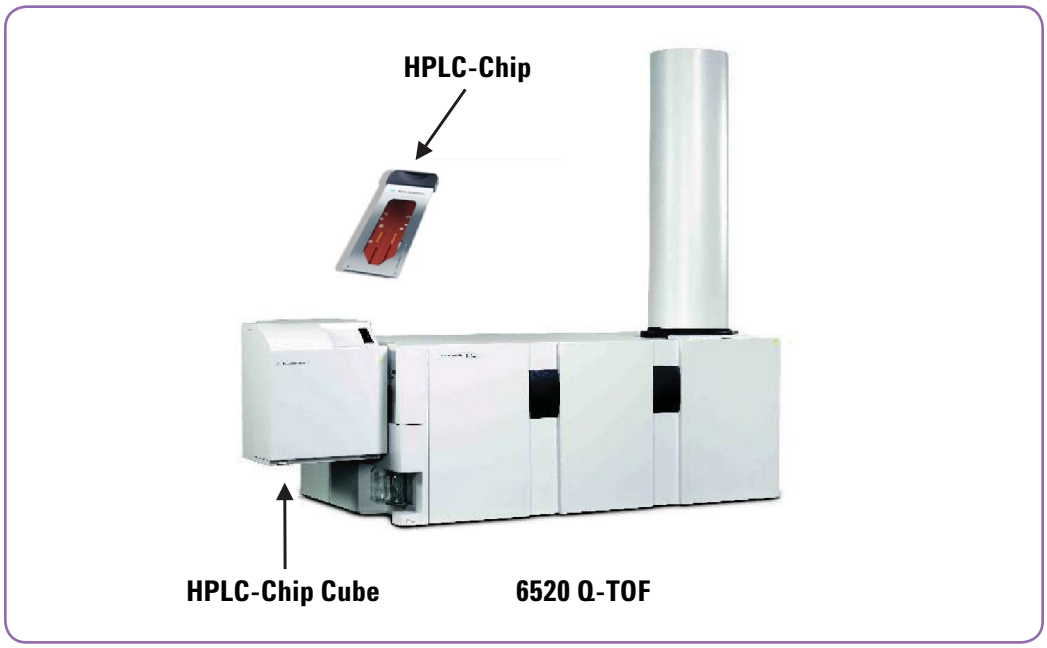


Figure 1. Agilent's HPLC-Chip technology coupled to a 6520 Accurate-Mass Q-TOF MS instrument.

Sample loading: With cap pump at 3% B.

Sample analysis: Gradient with nano pump as shown below.

MS parameters

Spectra were recorded in positive ion mode and in profile mode on the Agilent 6520 Accurate-Mass Q-TOF MS instrument.

Time (minutes)	B (%)
Initial	3
1	25
4	60
8	80
12	95
15	5

Stop time: 15 min

Vcap: 1750 V and drying gas flow of 5 L/min at 325°C was used.

Fragmentor voltage: 420 V for intact antibody/deglycosylated form and 350 V for the fragments.

Data were acquired in standard (20,000 m/z), 1 GHz, MS only mode, range 500-4,000 m/z (for fragments of mAb); 1,000-4,000 m/z (for intact mAb).

Data analysis

AgilentMass Hunter Qualitative Analysis software and the MassHunter BioConfirm software (version B.02.00) were used. Protein and fragment masses were obtained using maximum entropy deconvolution. Average masses for intact mAb, light chain, heavy chain, Fab, and Fc were calculated from sequences using the Sequence Editor feature found in the BioConfirm software.

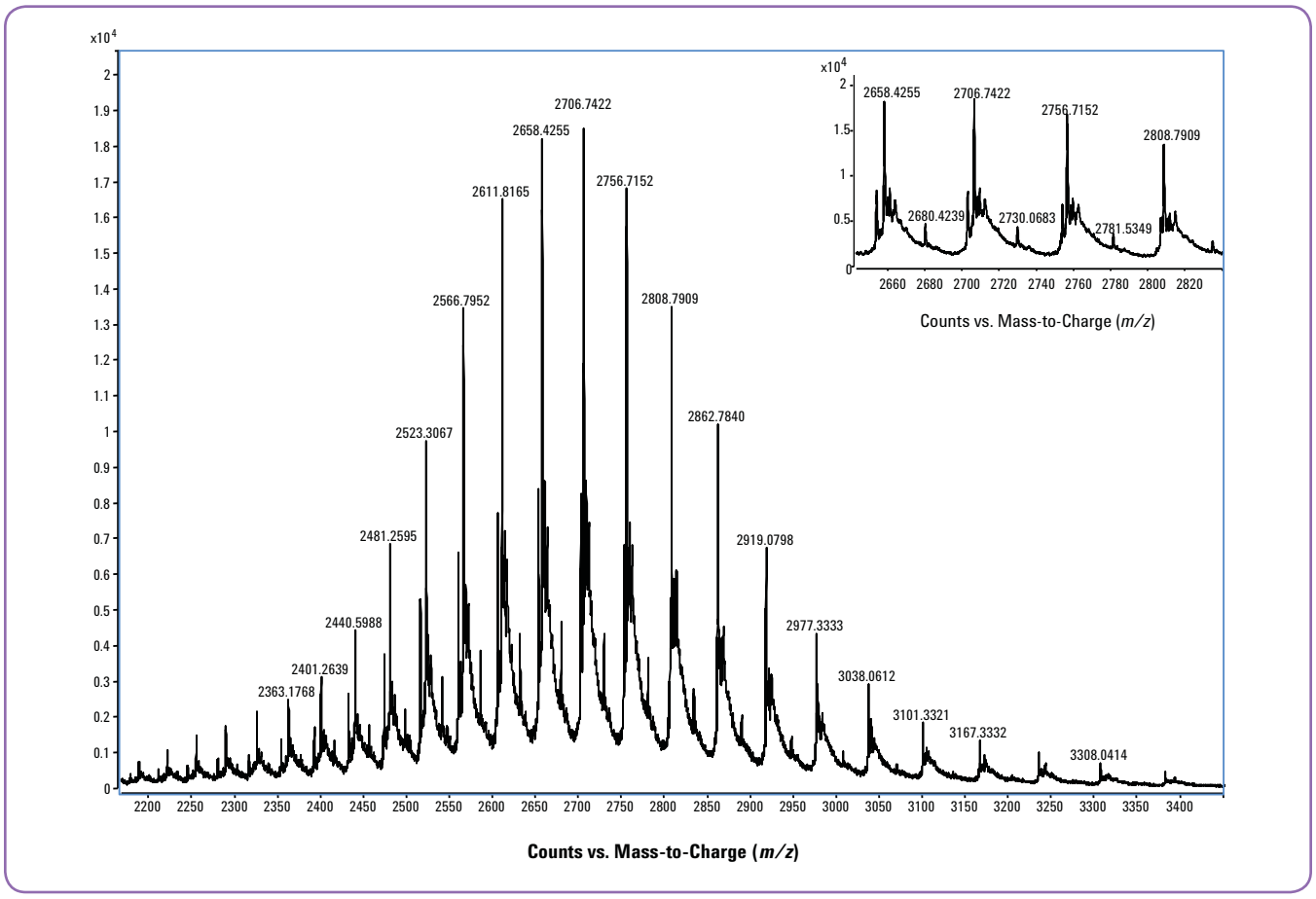


Figure 2. Mass spectrum of intact antibody with inset showing the expanded view of charge states of the antibody.

Results and Discussion

Analysis of Intact Antibody

Figure 2 shows the averaged mass spectrum of the intact antibody after elution from the HPLC-Chip. A very well distributed series of peaks is seen corresponding to the m/z of the many different charge state species of the intact antibody. Small satellite peaks within each charge state are also observed (see inset), corresponding to either adducts or modifications of the mAb. To gain more insight into the possible modification/adduct, deconvolution of the intact mass spectrum was performed using a maximum entropy algorithm (**Figure 3**).

The deconvoluted spectrum shows three major mass peaks at 148812.81 Da (calculated mass from sequence is 148811.95 Da; measured mass accuracy of 5.7 ppm), 147367.94 Da, and 145922.00 Da. The mass differences between the peaks are marked in **Figure 3.** The observed mass difference of 1444.87 Da (calculated mass=1445.35 Da) is attributed to one unit of the glycan (G0F) attached to the mAb. **Table 1** lists some of the commonly found N-linked glycans in the immunoglobulin. The other observed mass difference of 2890.81 Da (calculated mass=2890.7 Da) corresponds to two such units of G0F glycan, indicating that the mAb sample has three species: intact mAb with a pair of G0F glycans (148812.81 Da), mAb with one unit

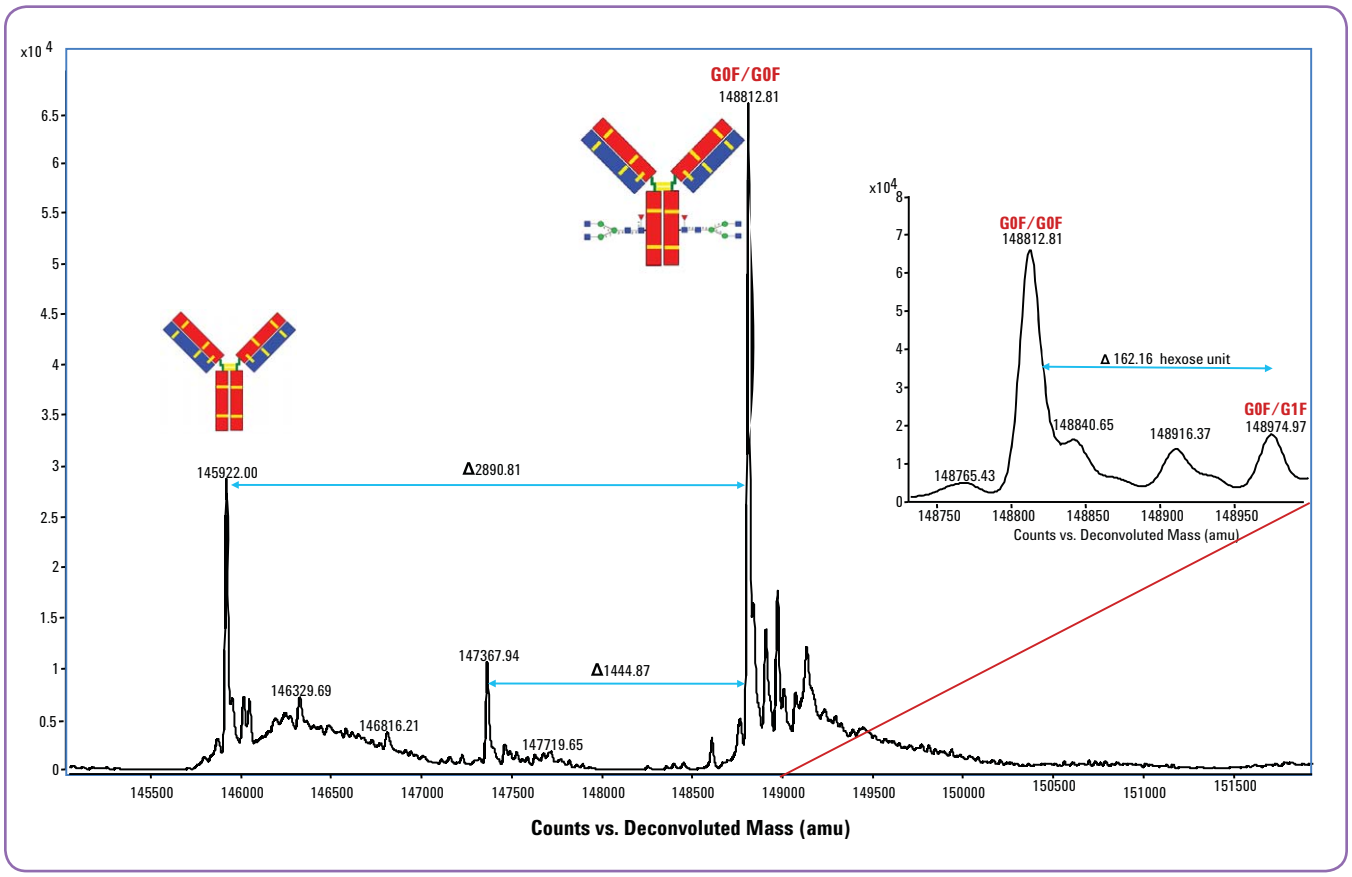
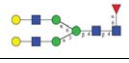


Figure 3. Deconvoluted spectrum of intact antibody with inset showing the expanded view of a small amount of G1F (addition of hexose unit to G0F, see Table 1) of the mAb.

of G0F glycan (147367.94 Da), and mAb devoid of any N-linked glycan part (145922.00 Da). Another peak **(Figure 3)** at 148974.97 Da was assigned to the addition of a hexose unit to the G0F glycan part of the antibody (observed mass increase of 162.16 Da; calculated mass is 162.14 Da), denoted as G1F in **Figure 3 (see Table 1)**.

Analysis of deglycosylated antibody

To confirm the assignment of the peak mass for the unglycosylated species at mass 145922.00 Da, a deglycosylation reaction of the mAb was performed. The mass spectrum of the deglycosylated mAb is shown in **Figure 4**. The spectrum shows well-defined charge state species with minimal adducts or satellite peaks. The deconvoluted spectrum **(Figure 5)** has a single molecular species at 145924.41 Da (calculated mass

Code	Oligosaccharide structure	Average mass	Comments
G2F		1769.64	
G1F		1607.49	Small amount found in this mAb
G0F		1445.35	Major form found in this mAb
G0		1299.21	

Galactose
Mannose
Fucose
N-acetylglucosamine

Table 1. Structures of N-linked glycans commonly found in IgG molecules [2] .

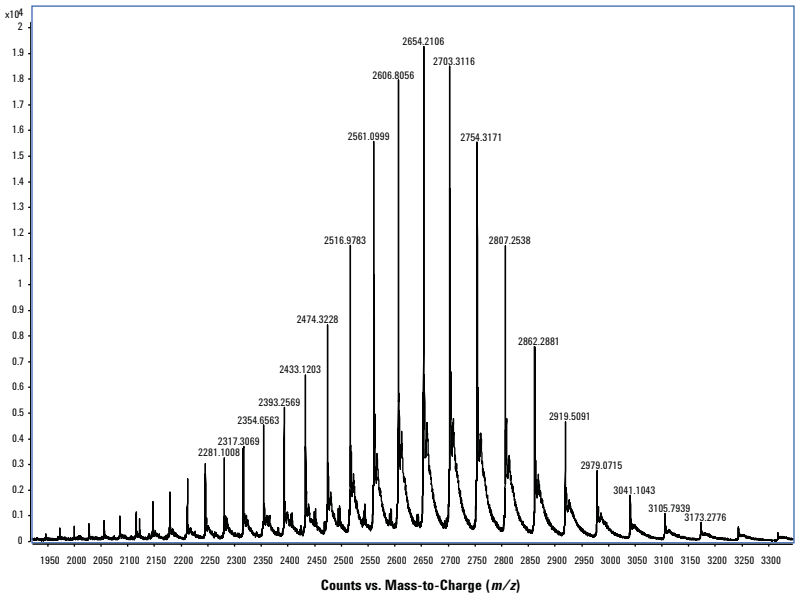


Figure 4. Mass spectrum of deglycosylated antibody.

from sequence, and accounting for N to D conversion which occurs during deglycation reaction, is 145923.24 Da; mass accuracy obtained is 8 ppm), indicating removal of all glycan moieties from the mAb. The mass obtained is also in close agreement with the mass assignment for the unglycosylated form (145922.00 Da) of the mAb in **Figure 3.**

Analysis of reduced antibody

To obtain more insight into the structure of the mAb, complete reduction using DTT was performed. The deconvoluted spectra of the light and heavy chains of the mAb are shown in **Figure 6**. The light chain has a measured mass of 23746.5 Da (calculated mass from sequence is 23746.63 Da; mass accuracy obtained is -5.45 ppm) and the heavy chain has a measured mass of 50675.58 Da (calculated mass from sequence is 50675.68 Da; mass accuracy obtained is 1.9 ppm).

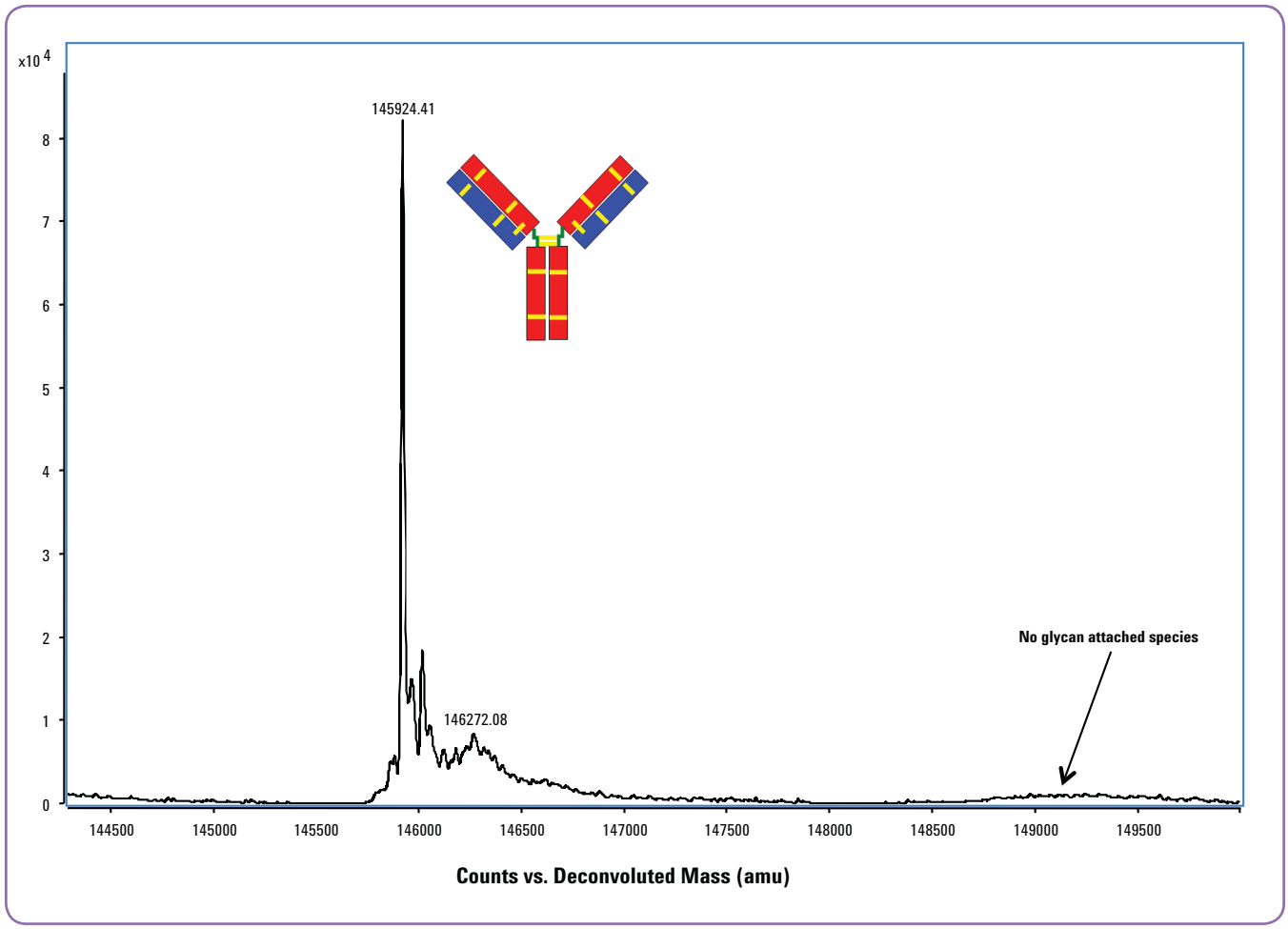


Figure 5. Deconvoluted spectrum of deglycosylated antibody.

Analysis of Fc and Fab fragments generated by papain digestion

Papain is used to generate Fab and Fc fragments from a mAb for various biotechnological/therapeutic applications. These fragments are also useful for additional structural characterization of a mAb. The mass spectra and deconvoluted spectra for Fc and Fab fragments are shown in **Figures 7 and 8**, respectively. **Figure 7B** further validates glycan attachment for the intact mAb.

The Fc fragment has a measured mass of 52755.64 Da (calculated mass from sequence is 52755.62 Da; mass accuracy ob-

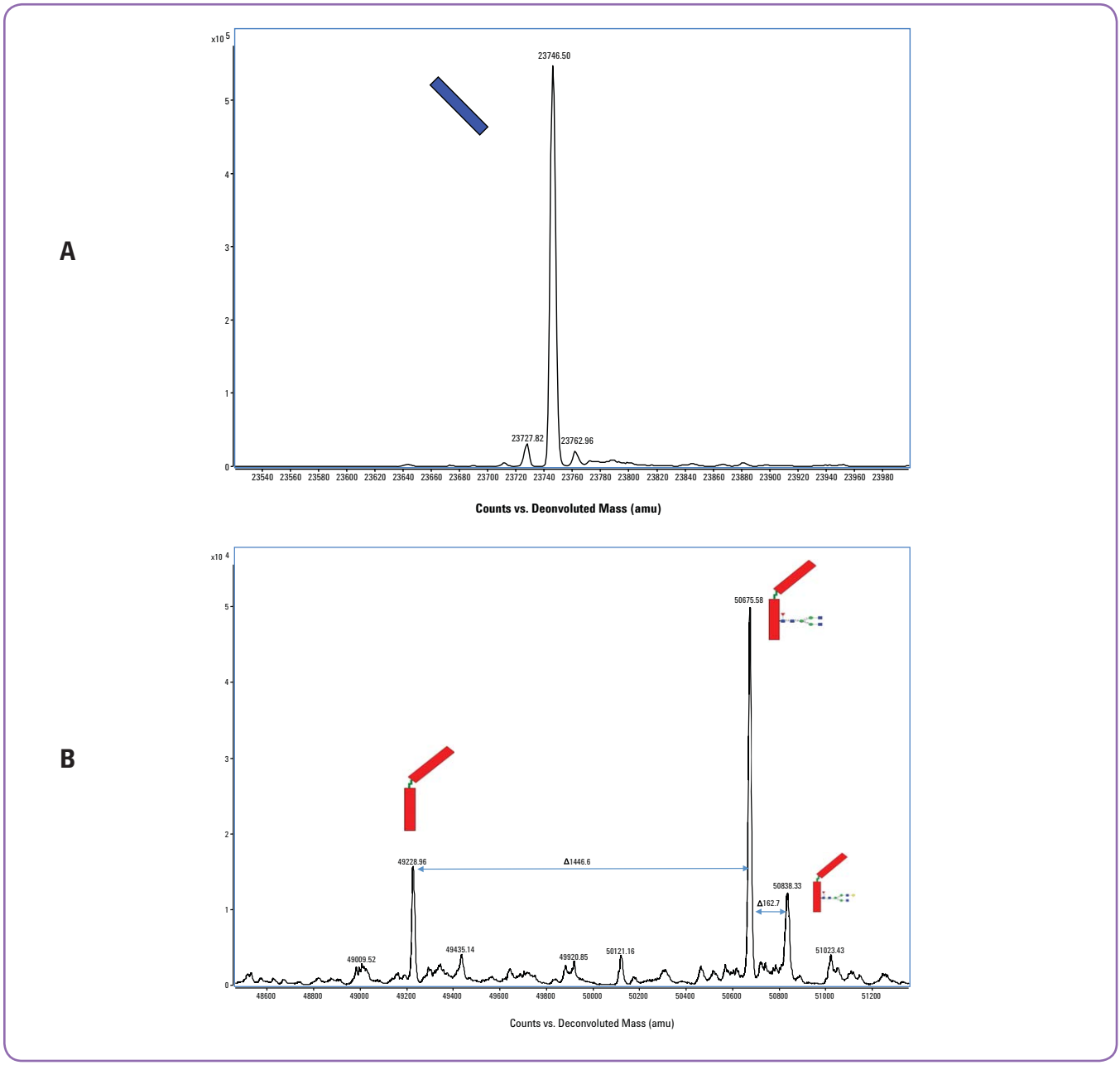


Figure 6. A) Deconvoluted spectrum of light chain of mAb (shown as blue bar).
B) Deconvoluted spectrum of heavy chain of mAb (shown as red bar with different sugar attachment).

tained is 0.38 ppm) and the Fab fragment has a measured mass of 48046.09 Da (calculated mass from sequence is 48046.18 Da; mass accuracy obtained is -1.9 ppm).

Low sample consumption

In our analyses, the amount of sample consumed was in the low nanogram level. The HPLC-Chip LC/MS method consumes significantly less sample compared to other LC methods, as shown in **Table 2**, making it well suited for the analysis of small amounts of biotherapeutics during the R&D stage or during product release.

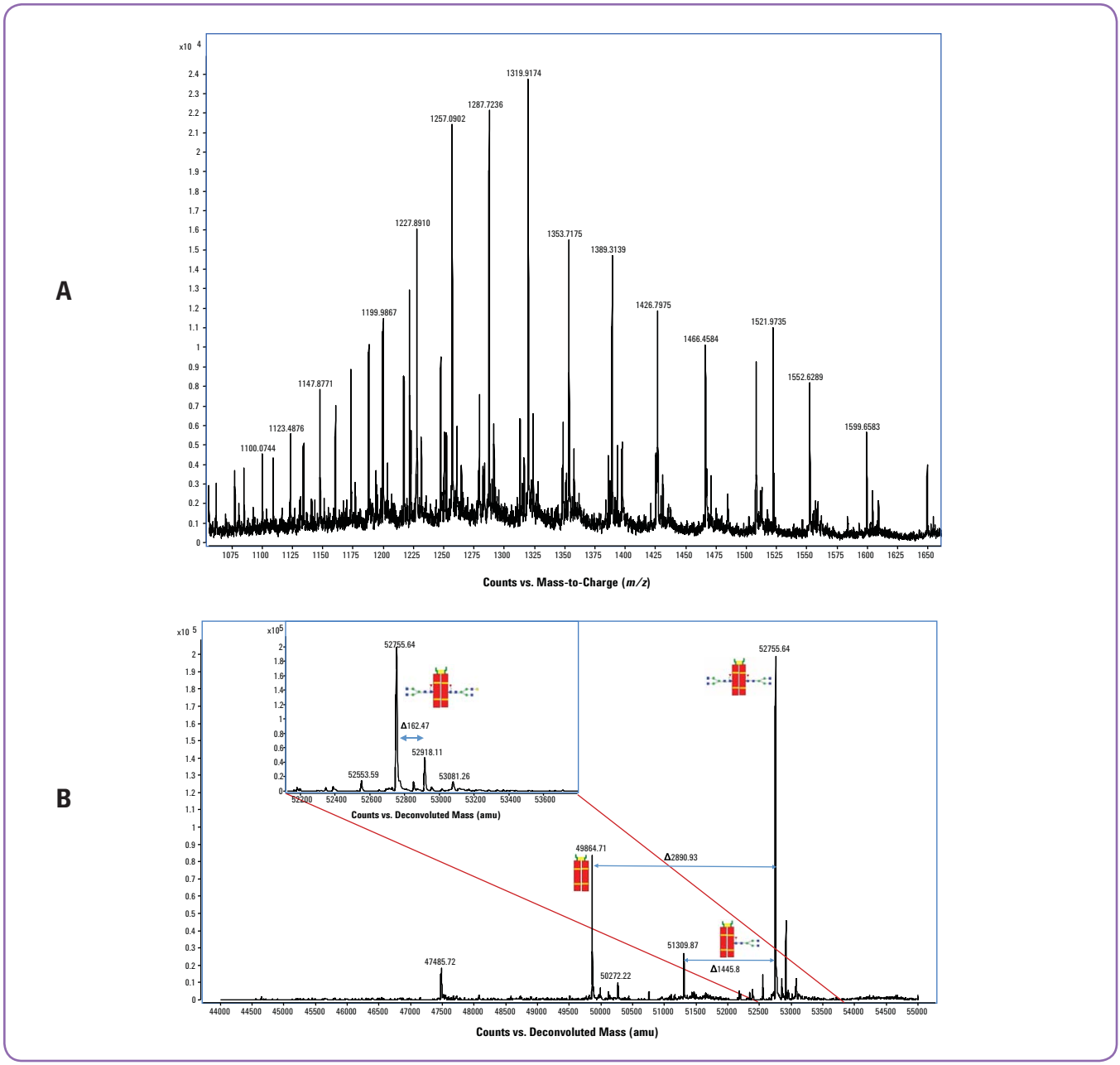


Figure 7. A) Mass spectrum of Fc fragment of mAb.
B) Deconvoluted mass of Fc fragment of mAb; inset shows the expanded view of small peak.

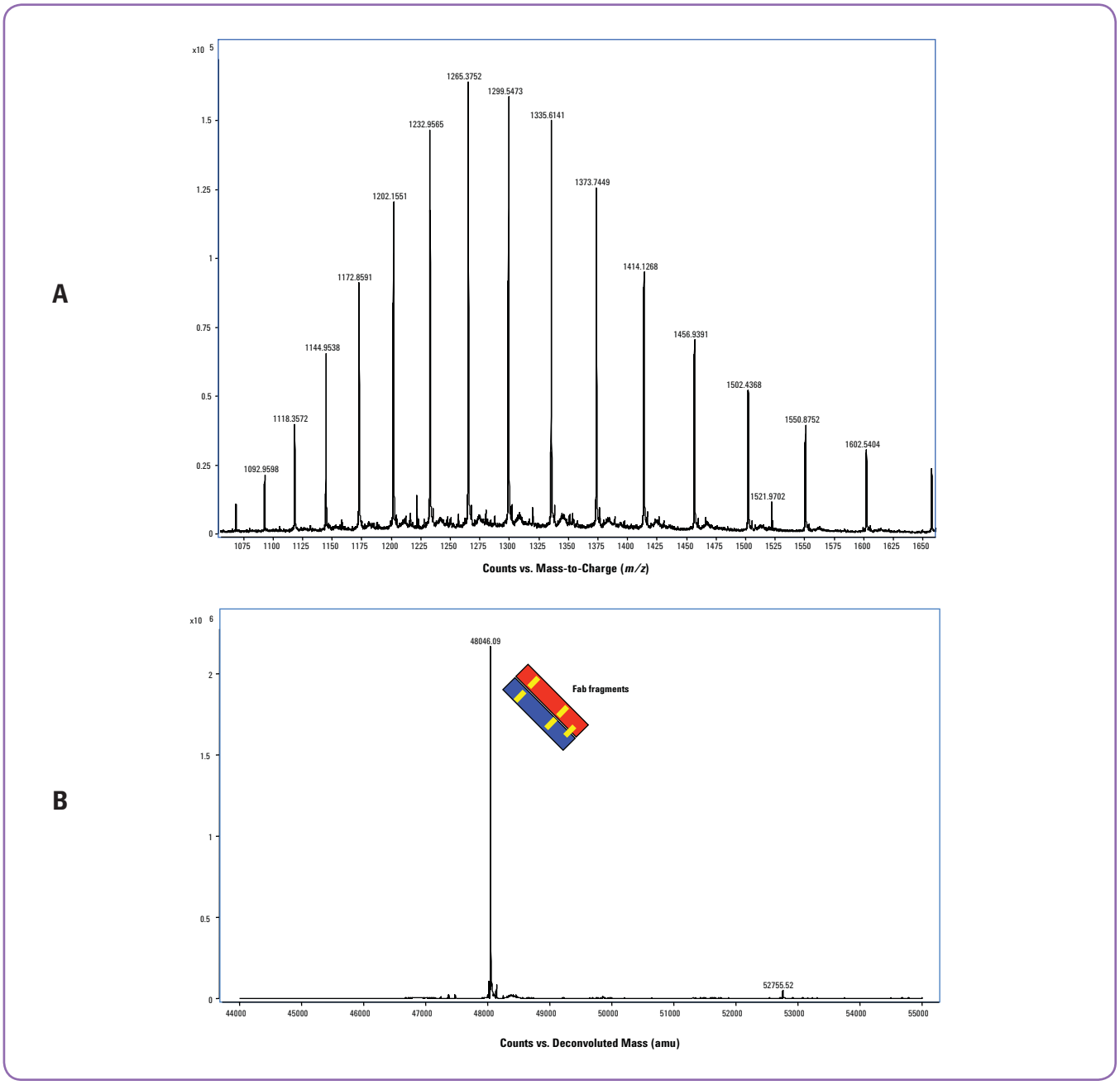


Figure 8. A) Mass spectrum of Fab fragment of mAb.
B) Deconvoluted mass of Fab fragment of mAb.

Column(dimension)	Amount of sample injected onto column	Reference
HPLC-Chip (43 × 0.075 mm)	5-10 ng	This note and [4]
Cap LC,C8 (50 × 1 mm)	~ 4 µg	[3]
Poroshell C8 (75 × 2.1 mm)	~200 ng	[4]

Table 2. Sample load on different columns.

Conclusions

Rapid and sensitive characterization of an intact mAb and its fragments at low nanogram levels has been demonstrated using Agilent's microfluidic HPLC-Chip/MS system coupled to the Agilent 6520 Accurate-Mass Q-TOF MS platform. The high sensitivity and sub-10 ppm mass accuracy of the nanoflow LC/MS system enabled identification of mAb heterogeneity, confirmation of mAb deglycosylation, and identification of Fab and Fc fragments. This approach is ideal for the routine analysis of small amounts of expensive and valuable biopharmaceuticals.

References

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4. Dayin Lin and Hongbin Liu, *Protein Society* 2008 Poster 236, HPLC-Chip QTOF Analysis of MAb.

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